Hydrogen Sulfide Mitigates Reperfusion Injury in a Porcine Model of Vascularized Composite Autotransplantation

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Background: Devastating extremity injuries are prevalent but often survivable on the modern battlefield. These complex injuries require advanced methods of reconstruction, involving prolonged ischemic periods and reperfusion injury. Using our group's validated porcine model of gracilis myocutaneous flap transplantation, this study demonstrates that an interim perfusion of hydrogen sulfide (H_2S) mitigates the effects of reperfusion injury in the setting of delayed restoration of blood flow.

Methods: A gracilis myocutaneous flap (200–400 g; surface area, 250 cm^2) was procured from the hind limb of a Yorkshire swine (70–90 kg, n = 16). The right external carotid artery and the internal jugular vein are the recipient axis. Group 1 (control, n = 6) underwent delayed anastomosis with a 3-hour ischemic period. Group 2 (n = 10) underwent a similar delayed anastomosis with an interim perfusion of H_2S during the ischemic period. The animals survived for 14 days. Systemic biomarker assays for skeletal muscle tissue injury (creatine kinase, lactate dehydrogenase, and aspartate transaminase) and proinflammatory markers (tumor necrosis factor α and interleukin 6) provide assessment of reperfusion injury at the cellular level.

Results: The control animals (3 hours of ischemia with an interim perfusion of heparinized saline) demonstrated increased levels of injury biomarkers and proinflammatory cytokines compared with the animals receiving H_2S infusion and identical ischemic interval. The control flaps had a mean creatine kinase level of 280×10^3 U/L ($\pm 80 \times 10^3$), compared with the H_2S group, which had a mean of 99×10^3 U/L ($\pm 14 \times 10^3$; $P = 0.0007$ at postoperative day 2). Lactate dehydrogenase levels (mean) were 26×10^3 U/L ($\pm 8 \times 10^3$) versus 9×10^3 U/L ($\pm 3 \times 10^3$; $P = 0.0004$) and aspartate transaminase levels (mean) were 1651 U/L (±324) versus (873 U/L [±279]; $P = 0.0013$) for the control and treatment groups, respectively. Similarly, an intergroup difference in IL-6 was found, although not statistically significant. Tumor necrosis factor α levels (mean) were 93 pg/mL (±14) versus 39 pg/mL (±4; $P = 0.0013$) for the control and treatment groups, respectively.

Conclusions: This study demonstrated the mitigating properties of H_2S on reperfusion injury. Interim perfusion with H_2S resulted in diminution of ischemia-dependent biomarkers after 3 hours of ischemia. Follow-up studies will translate these findings as an evolving method for reconstructing previously unreconstructable injuries.

Key Words: hydrogen sulfide, gracilis myocutaneous flap, vascularized composite tissue autotransplantation, vascularized composite allotransplantation, porcine, ischemia and reperfusion injury, microvascular, microsurgery

(*Ann Plast Surg* 2014;72: 594–598)

Received April 12, 2013, and accepted for publication, after revision, September 24, 2013.

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Conflicts of interest and sources of funding: none declared.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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ISSN: 0148-7043/14/7205-0594
DOI: 10.1097/SAP.0000000000000021

Reconstructive transplantation, or vascularized composite allotransplantation (VCA), is a technique that can restore form and function for patients with previously unreconstructable injuries. However, the technical and ethical challenges with VCA technology have reduced its application to a relatively small number of patients. The systemic immunosuppression required confers significant morbidity, and even mortality, in supporting an allograft that can improve quality of life but is not lifesaving. The skin component of a VCA is highly immunogenic and, in combination with other components of the composite tissue (bone, muscle, and neurovascular structures), results in a graft that requires relatively greater levels of immunosuppression than that of a solid organ transplant.[1–3]

This group has initiated a series of studies, using a validated porcine model of autotransplantation, to investigate the potential of graft-specific treatments directed at tissue stabilization.[4] A similar model has been described in an allotransplantation setting, and it is accepted that this is a powerful model in studying treatments in the VCA field.[5] It is hypothesized that reducing the inflammatory milieu associated with ischemia-reperfusion injury (IRI) in transplantation will attenuate the resultant antigenic storm. Data from both clinical and preclinical trials in solid organ transplantation have shown that increased severity of the obligate IRI at the time of transplantation, due to increased duration of cold ischemia, is associated with increased rates and severity of acute rejection.[6,7] Similarly, increased rates of acute rejection are associated with increased rates of chronic rejection, although the latter has yet to be described in the comparatively young field of VCA.[8] It is attractive to hypothesize that this reduction in immunogenic response may dampen allorecognition and, in turn, reduce immunosuppression requirements in the setting of VCA.

Various substances have been used in an attempt to ameliorate the effects of reperfusion injury on transplanted tissues; however, none have proven ideal for use in the clinical settings.[9] One substance of note, hydrogen sulfide (H_2S), has been used in small and large animal studies and has been demonstrated to hold significant promise as a cytoprotective agent when administered in appropriate doses.[9] hydrogen sulfide is the third gasotransmitter to be discovered, alongside nitrous oxide and carbon monoxide. In common with the other two, H_2S was originally thought simply to be a toxic gas.[10] However, studies have shown that H_2S is produced locally in the tissues by the action of 3 enzymes and has protective effects by various mechanisms on tissues as diverse as the kidney, the lung, the brain, the liver, the heart, and the vasculature.[11] In addition to local effects, systemic application of H_2S via inhalation by experimental animals has induced a suspended animation-like state in mice[12] and ameliorated the effects of renal IRI in pigs.[13] Its effects on composite tissue transfer have not been investigated and, consequently, are the area that this study addresses.

This experiment evaluates the ability of H_2S to ameliorate IRI in a validated swine free-flap model.

METHODS

The animals were handled and cared for under institutional guidelines in compliance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, Washington, DC: National Academy

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 MAY 2014	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Hydrogen sulfide mitigates reperfusion injury in a porcine model of vascularized composite autotransplantation	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Villamaria C. Y., Fries C. A., Spencer J. R., Roth M., Davis M. R.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**5**	

TABLE 1. Flaps by Group

Group	*n*	Flaps That Did Not Survive 14 d
3-h Ischemia (control)	6	2
3-h Ischemia (H_2S)	10	3

Press, 1996) and the American Association for the Accreditation of Laboratory Animal Care. Experimental protocols were approved by the Wilford Hall Ambulatory Surgical Center Clinical Research Division Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of Good Laboratory Practices. The animals were entered into the study after a 5-day period of acclimation in the housing facilities of the Wilford Hall Ambulatory Surgical Center Clinical Research Division; Yorkshire swine (70–90 kg) were used for all experiments. All animals were inspected before the study by a veterinarian and monitored daily by a technician.

Thirty to 45 minutes before induction of anesthesia, the animals were sedated with ketamine (15–20 mg/kg) and atropine (0.04–0.4 mg/kg) administered intramuscularly by veterinary technicians. The animals were preoxygenated for 2 to 3 minutes by mask with 100% ambient oxygen. Anesthesia was initiated with 3% to 4.5% isoflurane in an air-oxygen mixture of 40% to 60%. The animals were orally intubated with an appropriate size (6.0–7.5 mm) endotracheal tube. Anesthesia was maintained with 2% to 3% isoflurane in an air-oxygen mixture of 40% to 60% by a nurse anesthetist or a qualified surgical technician. The animal's heart rate, respiratory rate, oxygen saturation, and carbon dioxide levels were continuously monitored.

Sixteen animals were used, 6 in the control arm and 10 in the experimental arm (Table 1). Gracilis myocutaneous flap dissection and exposure of vessels within the neck were performed as per this group's validated model of free gracilis myocutaneous flap transfer in swine; the flaps weighed between 200 and 400 g, with a 250-g flap corresponding to a surface area of 250 cm^2. After exposure of neck vessels and procurement of the gracilis flap, the flap vessels were prepared for anastomosis within the neck cavity. In the treatment group (n = 10), the flaps were infused in an antegrade, intra-arterial fashion with H_2S solution (1 mL of 11.6-mg/mL solution per 400 g [weight of flap]). The venous effluent from the initial infusion was collected and was then infused again in the same antegrade fashion for 5 cycles to ensure tissue saturation. Once completed, the flaps underwent delayed inset and reperfusion (3 hours of ischemia). The control flaps (n = 6) similarly underwent 3 hours of ischemia. During the ischemic period, the tissue composites were kept at 4°C. The right external carotid artery and the internal jugular vein serve as the recipient axis. A surgical microscope (Leica F20, Wetzlar, Germany) was used for all microsurgical anastomoses.

The venous anastomosis was performed first with the use of a 2.5-mm venous coupler (Synovis GEM FLOW COUPLER, St Paul, Minn). Once the venous anastomosis is completed, the arterial anastomosis is performed with 2 running hemicircumferential 8.0 nylon sutures. The vascular clamps are then removed, and perfusion is reestablished. Once reperfusion is confirmed, blood is drawn systemically. The venous coupler wire remains attached to the external monitor while the neck incision is closed to ensure that there is no positional obstruction of venous flow. The neck incision is closed in layers.

Anesthesia is weaned, and the animal is extubated. During the postoperative survival period, the animals were housed in individual runs with freedom of movement and positioning. On postoperative days (PODs) 1, 2, 7, and 14, the animals were sedated with ketamine (15–20 mg/kg) and placed supine on the operating table for reassessment. Blood samples were collected and assayed for systemic biomarkers (complete blood count, chemistry, arterial blood gases, lactic acid, creatine kinase [CK], lactate dehydrogenase [LDH], and aspartate transaminase [AST]) and cytokines (tumor necrosis factor α [TNF-α] and interleukin 6 [IL-6]). The flap appearance and incisions are examined. Patency of the venous anastomosis is assessed via external attachment of the venous FLOW COUPLER. Sixteen animals were used for this study.

Statistical Analysis

The experimental design of this study was a mixed-effects, randomized complete block design with repeated measures. Subject was a random effect because the subjects were a sample randomly selected and randomly assigned to a group. Fixed effects were ischemic period, treatment group, and time of repeated measures because these effects cannot be generalized to other treatments and times.

Sample means and SEs were calculated for continuous dependent variables and were stratified by group. Frequency distributions, by group, were determined for categorical variables. Main effects (group, time, and group by time) for continuous data were tested (at $\alpha = 0.05$) using a mixed-effects repeated-measures multivariate analysis of variance. The Bonferroni method was used to correct the level of significance for multiple comparisons while investigating significant main effects. For categorical variables, frequency distributions by group were compared using the Fisher exact test ($\alpha = 0.05$). Analyses were performed using the SASR 9.2, SAS Institute, Inc, Cary, NC.

RESULTS

The biomarker and cytokine levels on PODs 1, 2, 7, and 14 demonstrated the effects of IRI at the cellular level. creatine kinase, LDH, and AST were compared because these enzymes are prevalent in the skeletal muscle and routinely measured as markers for reperfusion injury.[14,15] Similarly, TNF-α and IL-6 levels were assessed because these cytokines are crucial in initiating, sustaining, and regulating the inflammatory processes.[16,17]

The 10 animals that underwent treatment with H_2S after initiation of the 3-hour ischemic period were compared with the control animals that underwent a similar ischemic interval with interim perfusion of heparinized saline solution. Systemic levels of CK are maximal for both the control and treatment groups at 24 hours (Fig. 1). The control flaps subjected to 3 hours of ischemia had a mean CK level of 280×10^3 U/L ($\pm 80 \times 10^3$), compared with the H_2S group, which had a mean of 99×10^3 U/L ($\pm 14 \times 10^3$). The difference was statistically significant on POD 2 ($P = 0.0007$). After POD 1, the CK levels for both groups asymptomatically returned toward baseline through POD 14.

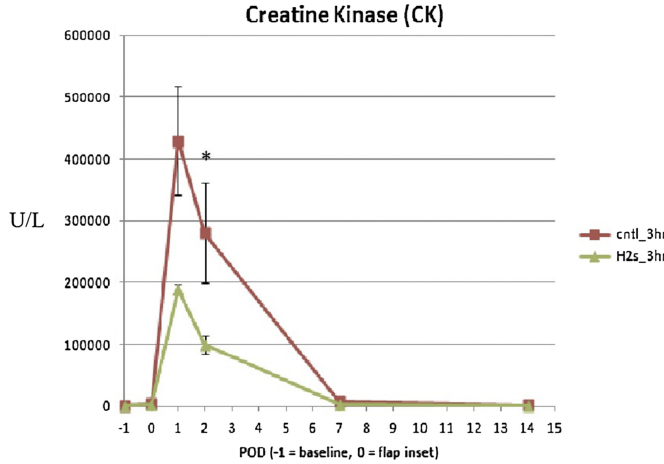

FIGURE 1. creatine kinase levels during the experimental course; *$P = 0.0007$.

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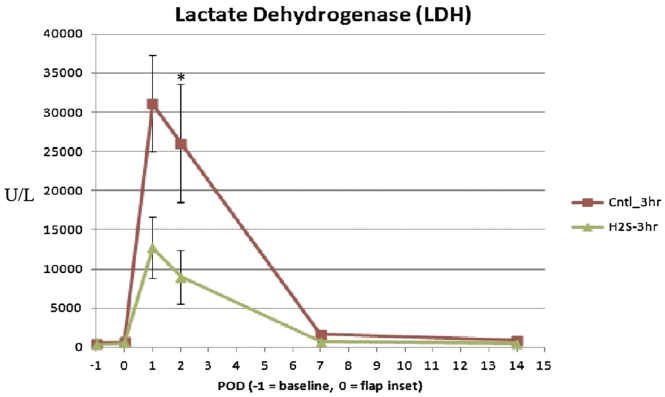

FIGURE 2. Lactate dehydrogenase levels during the experimental course; *$P = 0.0004$.

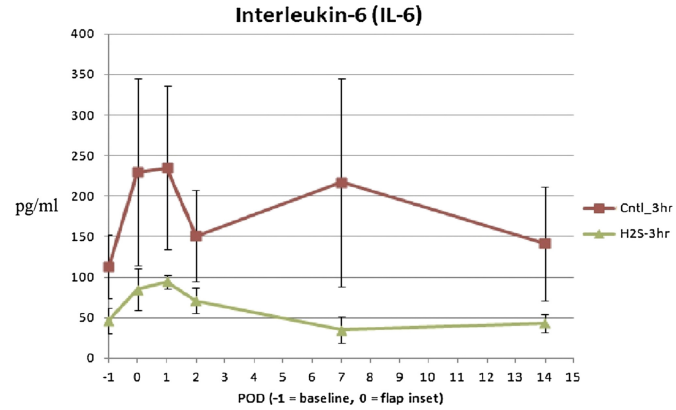

FIGURE 4. Interleukin 6 levels during the experimental course; $P > 0.05$.

The LDH levels in both groups demonstrated peak levels within 24 hours of operation. Similar to CK, there was a statistically significant difference between the mean LDH levels between the 2 groups at POD 2. The mean LDH for the 3-hour ischemic control group was 26×10^3 U/L ($\pm 8 \times 10^3$) versus 9×10^3 U/L ($\pm 3 \times 10^3$; $P = 0.0004$) in the group that received an interim perfusion of H_2S (Fig. 2).

Aspartate transaminase also demonstrates a statistically significant difference between the 3-hour control versus the H_2S group (1651 U/L [±324] vs 873 U/L [±279], respectively; $P = 0.0013$; Fig. 3).

There were also differences between the systemic levels of proinflammatory cytokines between the experimental groups. There is a difference when comparing the IL-6 levels between the control and treatment animals; however, these differences were not noted to be of statistical significance (Fig. 4). The difference in TNF-α levels between the groups, however, was found to be statistically significant at POD 1 and 7. On POD 1, the control group had a mean level of 68 pg/mL (±7), whereas the treatment group had a mean level of 30 pg/mL (±5; $P = 0.0010$). On POD 7, the TNF-α levels were 93 pg/mL (±14) and 39 pg/mL (±4; $P = 0.0013$) for the control and treatment groups, respectively (Fig. 5).

Eleven of 16 flaps survived for the duration of the protocol. Five flaps failed early, likely for technical reasons, and these animals were euthanized on POD 7; biochemical results from the flaps that did not last the full 14 days were included in the statistical analysis (Table 1). Postmortem analysis of the failed flaps showed that failure was due to technical failures of either the arterial or the venous anastomosis. Flap failure rates were not significantly different between the control and experimental groups.

The results of complete blood counts and blood chemistry showed no significant differences between the 2 groups.

By POD 14, the flaps from both control and H_2S groups showed good integration with the surrounding tissues.

DISCUSSION

Long-term (2-week) composite tissue viability was demonstrated in the setting of delayed reperfusion, with the effects of delayed restoration of blood flow ameliorated by H_2S. All flaps subjected to ischemia exhibited a significant rise in biomarkers when compared with baseline. The flaps treated with an interim perfusion of H_2S demonstrated a reduction in cellular lysis, reflected in a significantly blunted elevation of markers for skeletal muscle damage (CK, AST, and LDH). The effect of H_2S is such that a flap subjected to 3 hours of ischemia but treated with H_2S causes a smaller rise in the biomarkers when compared with the control flaps of both 1 and 3 hours of ischemic interval. A similar trend was also noted with the proinflammatory cytokines. The finding that the markers of muscle damage were relatively more affected than were the markers of inflammation suggests that the principal mode of action of H_2S may be cytoprotective, rather than reducing inflammation itself.

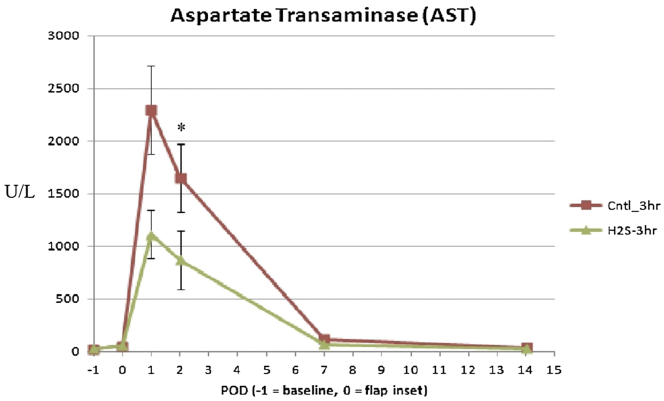

FIGURE 3. Aspartate transaminase levels during the experimental course; *$P = 0.0013$.

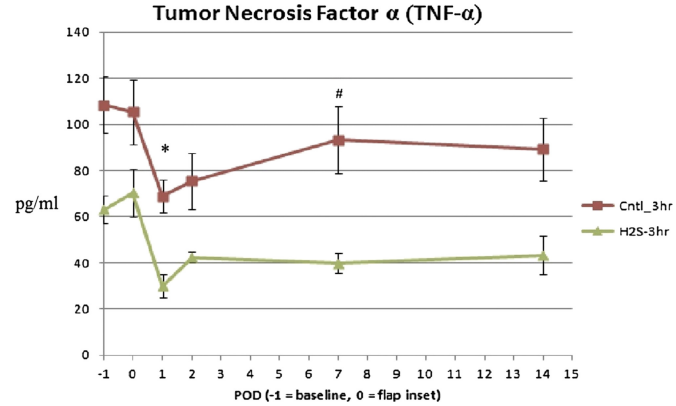

FIGURE 5. Tumor necrosis factor α levels during the experimental course; *$P = 0.0010$, #$P = 0.0013$.

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The effects of H_2S on the flaps in the longer term are not defined by this 14-day survival model, and no previous studies have used H_2S this way. However, in this study, the experiment flaps were well integrated clinically at the 14-day point, and the biochemical markers of cellular injury and inflammation had returned to normal levels. On the basis of the excretion of the compound, it is reasonable to expect that no further effects would be exerted on the transplanted tissue composite.

The safety of using H_2S is a potential concern particularly because it is clearly a toxic substance in higher doses, with the cytoprotective effects being exerted only at lower levels. Hydrogen sulfide is metabolized peripherally and renally excreted as sulphate compounds. The toxic effects of H_2S are exerted primarily on the nervous and the respiratory system. The Occupational Health and Safety Administration has reviewed the safety of H_2S and found that levels in excess of 10 ppm for 10 minutes are considered potentially harmful, based on effects on humans and animals after industrial or agricultural exposure.[18] Similar studies have found subtle effects on cellular immunity functions, which may have implications in the field of allotransplantation, including increases in levels of leukocytes, neutrophils, lymphocytes, $CD8^+$ lymphocytes, and complement C3.[19] It is beyond the scope of this experiment to establish mechanistic information of H_2S, and indeed, this is being actively investigated by the Defense Advanced Research Projects Agency. Imminent protocols transitioning H_2S into an allotransplant model will show what macro effects on immune rejection H_2S may have.

This study adds to a growing body of evidence relating to the role of H_2S in tissue preservation and stabilization. Blackstone et al[12] demonstrated the ability of H_2S to induce a state of suspended animation in mice by decreasing metabolic rate and oxygen requirements. Other groups have followed to investigate the cytoprotective effects of H_2S on skeletal muscle and solid organs subjected to the insults of ischemia and reperfusion injury. Henderson et al[9] demonstrated that pretreatment with H_2S conferred protection to muscle subjected to ischemia followed by a period of reperfusion. Simon et al[13] later examined the effects of H_2S on organ dysfunction in a porcine model of aortic occlusion–induced IRI. The study revealed that sulfide pretreatment attenuated renal damage, which corresponded with a less severe systemic inflammatory response. Data from our study corroborate previous findings that H_2S plays a role in attenuation of ischemia-reperfusion–induced injury. However, unlike previous studies, this is the first validation of its effect in a large animal using composite tissue blocks.

The authors believe that this is a robust model of composite tissue autotransplantation; however, limitations inherent in research using animal species must be taken into account. It is not possible in this experiment to demonstrate whether the differences in the biomarkers of reperfusion injury, demonstrated above, have clinical significance with respect to flap function or viability because there was no significant difference in composite tissue survival between the control and treatment groups or, indeed, to look at potential effects on the animal or the flap beyond 14 days. The flap failure rates within this model of 5 (31%) of 16 in all groups largely reflect the demands the flaps must withstand in the postoperative period. The animals were left untethered with full freedom of mobility in the postoperative period. Although the design of this model is to maximize flap viability and reliability as well as to limit morbidity, flap failure is predictably higher than that found in the clinical arena, wherein the free tissue transfer is protected and under strict surveillance.

The overt objective of this line of research is translation of the model to allotransplantation, in which the flap is transplanted into a second pig using the same technique. The principles designed to quell reperfusion injury by suppressing excessive antigen release secondary to oxidative and inflammatory injury will be important adjuncts to an allotransplantation model. Subsequent studies by this group will investigate whether the demonstrated effects of H_2S in reducing circulating biomarkers of reperfusion injury will translate to attenuation of acute rejection in VCA and reduce requirements for systemic immunosuppression.

Demands for extensive complex vascularized composite tissue reconstruction in the military and civilian settings call for improvements in the current techniques. Despite significant advances in microsurgical techniques in recent decades, prolonged ischemia and the resultant reperfusion injury remain a challenge in these complex reconstructions. Reperfusion injury results in a multifactorial inflammatory condition with both immediate and long-term effects.[20,21] Hydrogen sulfide was implemented as a measure to block the IRI process before its onset. This preemptive inhibition of inflammatory mediators may prevent the deleterious overstimulation of the inflammatory process and therefore ameliorate oxidative injury. Manipulation of the physiologic milieu before injury provides additional insight into reperfusion injury mechanisms and countermechanisms.

It is hypothesized that optimization of the postischemic physiologic milieu, by ameliorating the effects of reperfusion injury during vascularized composite tissue transfer, will offer advantages to the field of reconstructive allotransplantation. It may also enable free tissue transfer procedures to be performed in more critically ill patients, enabling earlier reconstruction with concomitant benefits in functional outcomes.

CONCLUSIONS

In the setting of delayed restoration of blood flow, H_2S demonstrated mitigating effects on reperfusion injury as evidenced by reduction of the systemic biomarkers of skeletal muscle tissue injury (CK, LDH, and AST) and proinflammatory cytokines (IL-6 and TNF-α). Follow-up studies will transition these findings to potentiate VCA.

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